



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US87/00924 (22) International Filing Date: 27 April 1987 (27.04.87) (31) Priority Application Number: 856,827 (32) Priority Date: 28 April 1986 (28.04.86) (33) Priority Country: US  (71) Applicant: ENDOTRONICS, INC. [US/US]; 8500 Evergreen Blvd., Coon Rapids, MN 55433 (US). (72) Inventors: MELINK, Georgiann, B. ; 1345 Berkeley, St. Paul, MN 55105 (US). SHANKAR, Raji, A. ; 1492 - 19th Avenue N.W., New Brighton, MN 55112 (US). (74) Agents: SAWICKI, Z., Peter et al.; Kinney &amp; Lange, 625 Fourth Avenue South, Suite 1500, Minneapolis, MN 55415-1659 (US).</p>		<p>(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i></p>
<p>(54) Title: METHOD OF CULTURING LEUKOCYTES</p> <div data-bbox="300 1239 1364 1585"> </div> <p>(57) Abstract</p> <p>A method for culturing leukocytes includes the culturing of leukocytes with interleukin-2 in a hollow fiber cartridge perfusion system (16) for at least four days to 19 days to achieve a harvest yield of at least 100% to 5000%. The leukocytes are recovered and have a lytic activity comparable to that of cells grown in a static culturing system.</p>		

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METHOD OF CULTURING LEUKOCYTESBACKGROUND OF THE INVENTION1. Field of the Invention.

05 The present invention relates to in-vitro  
culturing of purified human leukocytes, and in  
particular, the present invention relates to a method  
of culturing of purified human leukocytes in a  
perfusion culturing system.

2. Description of the Prior Art.

10 Attempts to manipulate the immune system  
with adoptive immunotherapy or by other means has had  
a long history. A very large number of experimental  
protocols have been tried in which agents thought to  
enhance and/or increase immunity have been given to  
15 patients. Most of these trials have not been  
successful and in the few cases in which success has  
been reported, it has been difficult to reproduce the  
successful aspects of the trial.

Adoptive immunotherapy in the contents of  
20 the present application involves the administration  
of immunologically active (immunocompetent) cells to  
an individual. These immunocompetent cells are taken  
either from the individual to be treated or from  
another individual. The purpose of administering  
25 immunocompetent cells to an individual is to provide  
a beneficial effect to the patient. For example, in  
the case of a cancer, cells are provided for the  
purpose of regressing and/or destroying a cancerous  
tumor.

30 Adoptive immunotherapy has been attempted by  
transferring immunocompetent cells from healthy  
animals to animals with a cancerous tumor. As such,

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animal experiments have suggested that an anti-tumor effect can be obtained with a high degree of antigen-specificity in certain tumor models. It has been found that the anti-tumor effect has been limited to certain tumors; and given the antigens-specificity of the effect, it has been assumed that in those cases (where antibodies have been ruled out as the effect or an important media of the effect) that leukocytes which include lymphocytes were involved.

More recently, these leukocytes have been described with reference to their anti-tumor activity and have been referred to as natural killer (NK) and lymphokine-activated killer (LAK) cells. (Other cells of the immune system which may be active in varying degrees regarding anti-tumor immunity also include cytotoxic T lymphocytes (CTL).)

NK and LAK cells are part of the immune system which preferentially lyse and/or kill target cells, including virally-infected and tumor cells. Rosenberg et al. have shown in animal models, as well as in man, that lymphocytes obtained from peripheral blood in man or spleen in mouse can be activated within and for a very few days with recombinant interleukin-2 (rIL-2), a factor that activated certain lymphocytes such as LAK cells. Rosenberg et al. has shown that LAK cells will have a regressive effect on tumors both in-vitro and in-vivo. This methodology has been applied to the treatment of cancer in man and encouraging results have been obtained in a significant number of patients,

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especially those with hypernephroma, melanoma and tumors of the colon.

However, a major difficulty in the protocol of Rosenberg et al. has been the growth of the number of cells required to obtain a therapeutic effect. To obtain a therapeutic effect in the patients where a regressive effect on the tumor has resulted in a cell dose between  $1 \times 10^{10}$  and  $2 \times 10^{11}$  LAK cells has been infected. Since cells in regular tissue culture (static culture) can only be grown at a maximum of approximately one million cells/ml, the amount of tissue culture medium, flasks, incubators and the like needed for the growth of these cells has been enormous. Further, the manipulation of the cells in terms of feeding, removal of waste from the medium and harvesting has been highly labor-intensive. This problem has limited the number of cell preparations that can be readied for treatment of patients and potentially limits the number of treatment centers that could provide such treatment to patients. In addition, the problem of culturing a sufficient number of cells is further intensified if a larger amount of cells would provide a more beneficial treatment and produce better results in patients.

#### SUMMARY OF THE INVENTION

The present invention is directed to a method of culturing leukocytes wherein the leukocytes are cultured in a perfusion system. Preferably, the leukocytes are cultured in an extracapillary space of a hollow fiber cartridge using a perfusion culturing system. The leukocytes are cultured for at least four days and up to 19 (optimal 12-16 days) providing

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a harvestable yield of a least 100% to 5900% recovery and having lytic activity at least equal to that of leukocytes cultured in a static culturing system. The cells are cultured with rIL-2 alone or rIL-2 and  
05 anti-CD3 monoclonal antibody. (Recent data suggests Anti-CD3 monoclonal antibody plus rIL-2 has been found to expand the cell number 10 fold greater than rIL-2 alone. A. Ochoa, et al.)

#### BRIEF DESCRIPTION OF THE DRAWINGS

10 Figure 1 is a diagrammatical view of a cell culturing system used in the method of the present invention.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention includes a method of  
15 culturing leukocytes in a perfusion system such that a harvestable yield of at least 100% to 5900% recovery of leukocytes having lytic activity is achieved.

By leukocytes is meant white blood  
20 corpuscles (cells) that combat infection.

By lymphocytes is meant those leukocytes without cytoplasmic granules. Lymphocytes normally number from 20% - 50% of total leukocytes and average 10-12 micrometers in diameter, but may be as large as  
25 20 micrometers. Lymphocytes are characterized by a deeply-staining, compact nucleus taking a dark blue. The nucleus occupies all or most of the cell, either in the center or at one side. The cytoplasm is usually clear, but in some cells bright  
30 red-disk-violet granules are seen.

By monocytes is meant a large mononuclear leukocyte having more protoplasm than a lymphocyte.

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By lympholines is meant those factors which, when presented to lymphocytes, activate the lymphocytes to lytic activity wherein the lymphokine-activated cells lyse and/or kill tumor cells. Examples of lymphokines are recombinant interleukin-2 (rIL-2), Beta-interleukin-1, Beta-interferon, alpha interferon and anti-CD3 monoclonal antibody.

By lytic activity is meant the ability of a cell to destroy a target cell or tumor.

The culturing of cells in hollow fiber cartridges is described in Knazek et al. U.S. Patents 3,821,087 and 3,883,393. However, limitations have been found in using the follow fiber cartridges of Knazek et al. in terms of achieving maximum cell density in the extracapillary space of the hollow fiber cartridge. Since large numbers of leukocytes are needed for adoptive immunotherapy treatment, high cell densities of leukocytes must be maintained viable and ready for use in such treatment.

The present invention includes the culturing and maintenance of lymphocytes in a hollow fiber cartridge perfusion culturing system such as is described in Patent Application Serial No. 658,549, filed on October 9, 1984 and entitled "Improved Hollow Fiber Cell Culture Device and Method of Operation," assigned to the same assignee as the present application, and which is hereby incorporated by reference.

Commercially available hollow fiber cartridge cell culturing systems are manufactured and marketed by Endotronics, Inc. of Coon Rapids,

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Minnesota, U.S.A., under the trademarks ACUSYST-P and ACUSYST-JR. This culturing system has been on sale for more than one year prior to the filing date of this application.

05           The ACUSYST-P cell culturing system is diagrammatically illustrated in Figure 1. The ACUSYST-JR is a scale down model of an ACUSYST-P, composed of one hollow fiber cartridge rather than 2-6. The system generally indicated at 10 includes a  
10   primary media circulation system 12 and a secondary media circulation system 14 for circulating medium within a hollow fiber cartridge 16. The circulation system 12 includes a primary medium supply 18 and a  
15   pump 20, preferably a bellows-type pump, connected by tubing 22. The pump 20 is fluidly connected by tubing 24 to the hollow fiber cartridge 16. Tubing 22 and 24 are designated as the supply side of the circulation system 20 supplying medium to the hollow  
20   fiber cartridge 16. Medium is circulated back to the supply source 18 by tubing 26. The circulation system 12 is fluidly connected to lumens of hollow fibers 28 of the hollow fiber cartridge 16 in a well known manner.

          The circulation system 14 supplies medium to  
25   the extracapillary space 30 of the hollow fiber cartridge 16, that space being defined as the space between the outer wall surfaces of the hollow fibers and the shell 32 of the hollow fiber cartridge. The circulation system 14 includes a supply source  
30   (expansion chamber 345) of medium that supplies medium to the extracapillary space 30 through tubing 36. Medium is returned to the supply source 34



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through tubing 38. The tubing 36 and 38, each have monodirectional valves in line so that medium flows in a direction as indicated by arrows 40 and 42.

05 The supply source 34 is kept at a constant pressure by supplying gas to the source 34. Typically, the gas pressure is kept constant at approximately 100 mmHg above atmospheric. Similarly, the supply source 18 is also pressurized by gas. However, the gas pressure is cycled in supply source 10 18 from 9 to 100 mmHg above atmospheric. The cycling of the gas pressure produces a changing pressure drop across the membrane walls of the hollow fibers and consequently provides a circulation of the medium within the extracapillary space of the hollow fiber 15 cartridge. Circulation of the medium within the extracapillary space of the hollow fiber cartridge provides for minimization of gradients of nutrients and waste products and minimization and/or elimination of microenvironments and anoxic pockets.

20 The system 10 is controlled by a digital computer system (not shown) which controls the pump 20 and the gas pressures in both supply sources 18 and 34 and the cycling of the gas pressure in supply source 18.

25 Using the above-mentioned perfusion system has resulted in yields of leukocytes of greater than 100% and up to 5900%. By yield is meant that value obtained by dividing the number of cells initially placed in the hollow fiber cartridge by the number of 30 cells harvested in a sterile condition useful for infusion into a patient. Prior to the present invention, using static culturing techniques, yields

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on the order of approximately 38%-82% were obtained. (Rosenberg et al.) Consequently, a lesser amount of cells were available for infusion to the patient than had been removed from the patient through leukapheresis. As is easily understood, subjecting an already weakened patient, for example, a patient suffering from cancer, to extensive leukapheresis is undesirable. Minimizing the leukapheresis by obtaining an equal or a greater amount of cells through culturing of the cells is highly desirable.

Adoptive immunotherapy is initially started by obtaining leukocytes from blood of the patient by subjecting the blood to leukapheresis. The leukapheresis cell suspension is then centrifuged and then separated leukocytes are harvested, washed and inoculated into the extracapillary space of the hollow fiber cartridge. The cartridges (from 1-6) are inoculated with an average of  $0.7 \times 10^9$  cells/cartridge (0.25 to  $2.2 \times 10^9$ /cartridge) and the cell culturing system activated to culture the cells.

The cells are cultured with recombinant interleukin-2 (rIL-2) obtained from Cetus Corporation of Emeryville, California to produce a lympholine-activated killer (LAK) cell. In a number of these experiments, cells were cultured with anti-CD3 (OKT3, Ortho Pharmaceutical, New Jersey) monoclonal antibody plus rIL-2.

The LAK cells are removed from the cartridges after culture and activation (4-19 days) and are then administered intravenously through a venous catheter or by direct infusion into an artery

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via a percutaneous catheter. The effects of such infusion can be observed on a cancerous tumor by techniques well known in the art. The administration of the LAK cells can be continued until the tumor has  
05 totally disappeared or until efforts show no further regression of the tumor. rIL-2 can also be administered in conjunction with the infusion of the LAK cells, depending upon the results observed as to the regression of the tumor and the effect of  
10 infusion of the LAK cells into the patient.

The following example is illustrative only and is not intended to limit the present invention. The example is submitted in order to demonstrate more explicitly the method of the present invention.

#### Example 1

15 The media used in the circulation loop providing media to the lumina of the hollow fibers was RPMI-1640 (Gibco, New York) containing 2.4mM L-glutamine (12 ml/L of a 200 mM solution), and 50  
20 ug/ml gentamycin (0.5 ml/L of 100 g/L solution) (Sigma, MO).

The circulation media for the circulation loop providing media to the extracapillary space included 454 ml of RPMI-1640 medium (Gibco, New York  
25 or Mediatech, VA), 40 ml human serum (filtered through .45 Um filter), 0.25 ml gentamycin, 6 ml L-glutamine, and recombinant interleukin-2 (rIL-2), 3000 U/ml, total units =  $1.5 \times 10^6$  units (Cetus Corporation of Emeryville, California). This  
30 recirculation media was exchanged every forty-eight hours with fresh recirculation media to replenish the nutrients and rIL2. 500 ml of the medium, having the

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same composition as the recirculation medium was used to coat the extracapillary space of the hollow fibers in preparation for inoculation of the leukocytes.

05 A unit of blood (approximately 200 ml) was obtained through leukapheresis. The red blood cells were separated from the white blood cells using a ficoll oypaque gradient protocol. The separation was performed in the V50 Haemonetics instrument.

10 The white cells were resuspended in complete RPMI-1640 media (Gibco, New York or Mediatech, VA) containing 8% human serum and 3000 U/ml rIL-2. The concentration of the cells is adjusted to  $0.5 - 4.4 \times 10^7$  cells per ml and the cells placed in two 60 ml syringes containing approximately 50 ml each of the  
15 solution. The cells are injected into the extracapillary space of the bioreactors.

The ACUSYST-P feed rate was set to 50 ml per hour. The hollow fiber cartridge was coated 2 to 24 hours prior to inoculation with 500 ml of the coating  
20 medium. The ACUSYST-JR is run identically to the ACUSYST-P, however, the volumes of media and cells used are proportional to the number of cartridges used.

One to six cartridges were inoculated with  
25 25 ml each of the cells from the two 60 ml syringes via the bypass line and the expansion chamber sample port. Each cartridge was seeded with  $0.25 - 2.2 \times 10^9$  cells having a viability no less than 95%. The viability of the cells was tested by Trypan Blue  
30 exclusion. In a number of experiments anti-CD3 (OKT3) was added to the coating buffer as well as the white blood cells prior to inoculation (10-100

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ng/ml). Forty-eight hours after inoculation the anti-CD3 antibody was removed from the system. Thereafter, the cells were cultured in the same fashion as that established for culturing cells with rIL-2.

05 The cells were cultured in a routine manner by cycling the pressure in the primary circulation system between 0 and 100 mmHg above atmospheric and keeping the pressure in the expansion chamber  
10 constant at approximately 100 mmHg above atmospheric. Samples were taken daily from the system to monitor pH, dissolved oxygen, glucose and lactate concentrations. These parameters were being maintained at an optimal set point by adjusting the  
15 media feed rate through the IC circuit (circulation system 12).

After culturing the cells from 12-16 days, the cells were removed from the extracapillary space of the cartridge using the V50 Haemonetics instrument  
20 (Braintree, MA) as a cell concentrator. Cells were flushed from the system with approximately 2 liter of normal saline supplemented with 1% human serum albumin and then concentrated to approximately 350 (250-500) mls. The cells were transferred to a 600  
25 ml IV bag (Travenol, IL). The entire procedure was performed in a sterile fashion.

From 35 culturing runs it was found that the average yields were 2000% (range 100-5900) after an average of 14 days of culture with rIL-2 alone or  
30 rIL-2 and OKT3. Overall viability after cell is removed 86% (63-94%).

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In one typical experiment where cells were cultured with rIL-2 alone, a yield of 2400% was obtained (see Trial #1). In another experiment (trial #2) where the cells were cultured with OKT3 plus rIL-2, a yield of 4500% was obtained. Both systems were cultured for fourteen days.

The yield figure is calculated based on the number of cells seeded in the cartridge and the total number of cells removed from the cartridge. In the cell culturing process, a number of cells are lost due to the circulation of the media through the extracapillary space in which the cells are being cultured.

The cells obtained from Trials I and II were tested for lytic activity and compared to cells cultured in a static culturing system. The static cells were cultured for 14 days in the same type of medium as the ACUSYST-P cultured cells, and maintained at less than  $1 \times 10^6$  cells/ml by subculturing. The target cells used in measuring lytic activity were HL-60, K562, Daudi and fresh tumor which are well known in the art. The results of these comparisons are listed in Tables 1 and 2.

The results in Tables 1 and 2 indicated that the cells cultured according to the present invention have comparable lytic activity as to cells cultured in a static culturing system. (It should be noted that some values have a standard deviation of greater than 10% and care should be taken in any conclusions made with these values.

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TABLE 1Trial 1

<u>Ratio of Effector Cells to Target Cells</u>	<u>Static Culture</u>	<u>Present Invention</u>
<u>HL60</u>		
30:1	27.4	58.8
10:1	17.1	53.8
3:1	9.7	40.6
1:1	5.6	26.0
0.3:1	2.3	13.7
<u>K562</u>		
30:1	56.7	60.3
10:1	53.1	55.2*
3:1	51.9	64.7
1:1	32.1	57.5
0:3	12.2	35.4
<u>Daudi</u>		
30:1	64.3	74.7
10:1	61.7	75.6
3:1	53.5	69.1
1:1	30.5	53.5
0.3:1	11.8	33.3
<u>Tumor</u>		
30:1	29.9	41.0
10:1	16.5	40.0*
3:1	6.7	38.1
1:1	5.1	19.0
0.3:1	1.4	10.0

\*SD is greater than 10%.

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TABLE 2Trial 2

<u>Ratio of Effector Cells to Target Cells</u>	<u>Static Culture</u>	<u>Present Invention</u>
<u>HL60</u>		
30:1	32.9	18
10:1	25.0	12.4
3:1	18.6	8.9
1:1	14.2	2.3
0.3:1	8.3	2.1
<u>K562</u>		
30:1	59.8	53.0
10:1	48.9	54.7
3:1	50.0	41.5
1:1	44.5	26.2
0.3:1	26.4	8.4
<u>Daudi</u>		
30:1	65.0	57.8
10:1	59.3	59.8
3:1	55.6	45.1
1:1	55.8	26.2
0.3:1	34.8	11.7
<u>Tumor</u>		
30:1	6.5	14.0*
10:1	2.7	5.4*
3:1	1.9*	3.5*
1:1	-2.7*	0.8*
0.3:1	-4.0*	6.3

\*SD is greater than 10%

Although the present invention has been described with reference to preferred embodiments, namely, leukocytes cultured in an ACUSYST-P or ACUSYST-JR and activated with OKT3 and/or rIL2 for an average of 14 days, workers skilled in the art will recognize that changes may be made in form and detail without departing from the spirit and scope of the invention.

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WHAT IS CLAIMED IS:

1. A method of culturing leukocytes comprising:  
culturing the leukocytes in a hollow fiber cartridge for at least four days to obtain a cell harvest having a yield of at least 100%.
2. The method of claim 1 wherein the leukocytes are cultured using a lymphokine.
3. The method of claim 2 wherein the lymphokine is recombinant interleukin-2 and or others.
4. The method of claim 1 wherein the leukocytes are cultured for approximately 4 to 19 days.
5. A protocol for immunotherapy comprising:  
culturing of leukocytes in a hollow fiber for at least four days to obtain a cell harvest having a yield of at least 100%.
6. The protocol of claim 5 wherein the leukocytes are cultured using a lymphokine.
7. The protocol of claim 6 wherein the lymphokine is interleukin-2 and/or others.
8. The protocol of claim 5 wherein the leukocytes are cultured for approximately 4 to 19 days.

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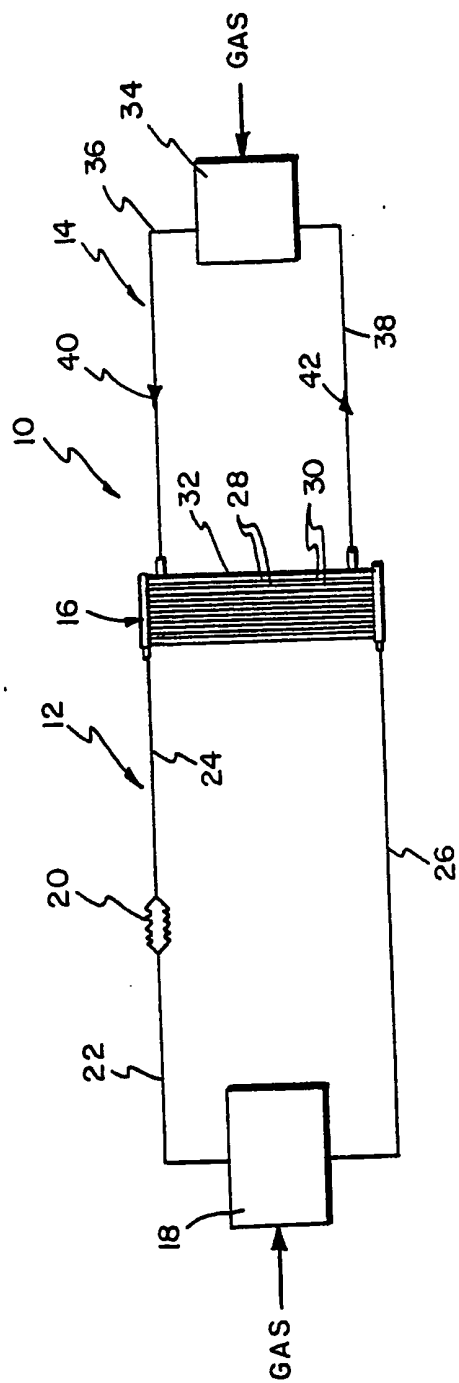


Fig. 1

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/00924

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(4): C12N 05/00		
US. CL: 435/240		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S.	435/240, 241; 424/93	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *		
CHEMICAL ABSTRACTS, BIOLOGICAL ABSTRACTS, LEUKOCYTE OR LYMPHOCYTE AND ADOPTIVE OR HOLLOW FIBERS		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> 14		
Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
Y	Science 225, 28 September 1984, MULE et al, "Adoptive Immunotherapy of Established Pulmonary Metastases with LAK Cells and Recombinant Interleukin-2" pages 1487-1489.	1-11
Y	U.S., A, 4,391,912 (YOSHIDA) 5 July 1983. See example 5.	1-11
Y	Cell 23 January 1981, NABEL et al, "Use of Cloned Populations of Mouse Lymphocytes to Analyze Cellular Differentiation", pages 19-28. See page 26.	1-11
<p>* Special categories of cited documents: 15</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 1	Date of Mailing of this International Search Report 2	
22 July 1987	11 AUG 1987	
International Searching Authority 1	Signature of Authorized Officer 19	
ISA/US	John Tarcza <i>John E. Tarcza</i>	